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PRINCIPAL INVESTIGATOR: Michael Ittmann MD PhD

CONTRACTING ORGANIZATION: BAYLOR COLLEGE OF MEDICINE
HOUSTON, TEXAS 77030

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Michael Ittmann MD PhD

E-Mail: mittmann@bcm.edu

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14. ABSTRACT

Prostate cancer (PCa) remains the most common malignancy and the second leading cause of cancer-related death for men in the United States. Recent studies have shown significant interactions between nerves and adjacent cancer cells that promote cell survival, proliferation and migration of PCa cells. Our studies of laser captured prostate cancer reactive stroma have shown that among the most upregulated genes is glial cell line-derived neurotrophic factor (GDNF), which is expressed by peripheral nerves. GDNF binds to RET, a receptor tyrosine kinase, in conjunction with its co-receptor GFRα1 (GFRA1) and activates cellular signaling. Both RET and GFRA1 are expressed on all PCa cell lines tested and RET protein is increased in PCa. Studies in pancreatic cancer strongly implicate GDNF as a key factor promoting perineural migration. We will test the hypothesis that GDNF is expressed by nerves and that it acts on RET/GFRA1 in adjacent PCa cells to promote proliferation and invasion and inhibit apoptosis and that disruption of this signaling cascade will inhibit PCa progression in vivo.

15. SUBJECT TERMS

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Table of Contents

1. INTRODUCTION.

Prostate cancer (PCa) remains the most common malignancy and the second leading cause of cancer-related death for men in the United States. The tumor microenvironment plays an important role in the initiation and progression of PCa. One important component of this microenvironment is nerves. PCa has a propensity to grow in perineural locations and large diameter perineural tumor is one of the most significant pathological predictors of poor outcome following radical prostatectomy. Perineural invasion is also associated with poor outcomes following radiation therapy. Recent studies have shown significant interactions between nerves and adjacent cancer cells that promote cell survival, proliferation and migration of PCa cells. Recent landmark studies have shown that chemical or surgical ablation of nerves inhibits tumorigenesis and metastasis in both xenograft and transgenic mouse models of PCa, unequivocally establishing that nerve-PCa cell interactions play a significant role in PCa initiation and progression. Interestingly, PCa cells and nerves have bidirectional interactions. Recent studies have shown that PCa induces axonogenesis and neurogenesis and higher nerve densities in the microenvironment are associated with aggressive clinical behavior in PCa. **Thus nerves and PCa cells interact in a potential feed forward loop in which nerves promote prostate cancer cell proliferation, survival and invasion and PCa cells in turn increase the number of nerve fibers via increased axonogenesis and neurogenesis**.

Our studies of laser captured prostate cancer reactive stroma[1] have shown that among the most upregulated genes is glial cell line-derived neurotrophic factor (GDNF), which is expressed by peripheral nerves. GDNF binds to RET, a receptor tyrosine kinase, in conjunction with its co-receptor GFRα1 (GFRA1) and activates cellular signaling. Both RET and GFRA1 are expressed on all PCa cell lines tested and RET protein is increased in PCa. Studies in pancreatic cancer strongly implicate GDNF as a key factor promoting perineural migration.

Our goal is test the hypothesis that GDNF is expressed by nerves and that it acts on RET in adjacent PCa cells to promote proliferation and invasion and inhibit apoptosis and that disruption of this signaling cascade will inhibit PCa progression in vivo.

2. KEYWORDS: prostate cancer; nerve, RET, GDNF, GFRα1
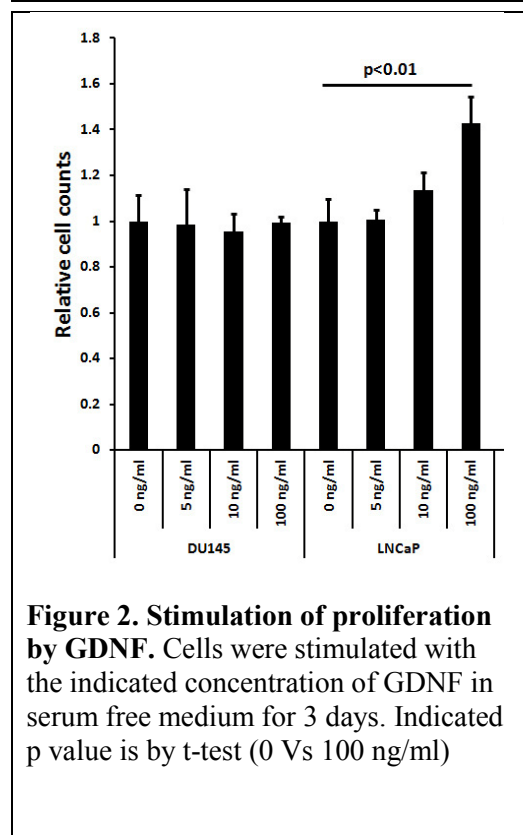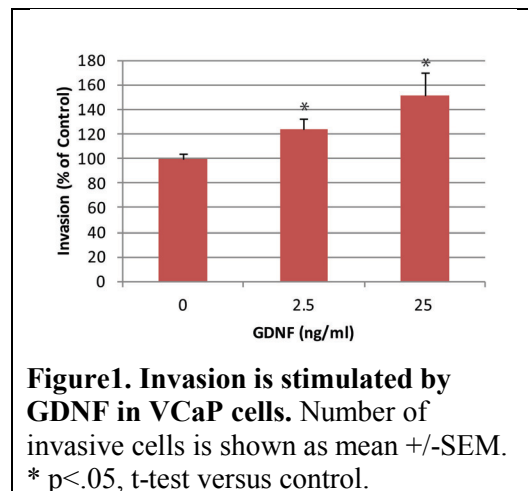
3. OVERALL PROJECT SUMMARY:

Major Task 1. Obtain regulatory approvals for animal and human protocols (Months 1-3)

All regulatory approvals have been obtained
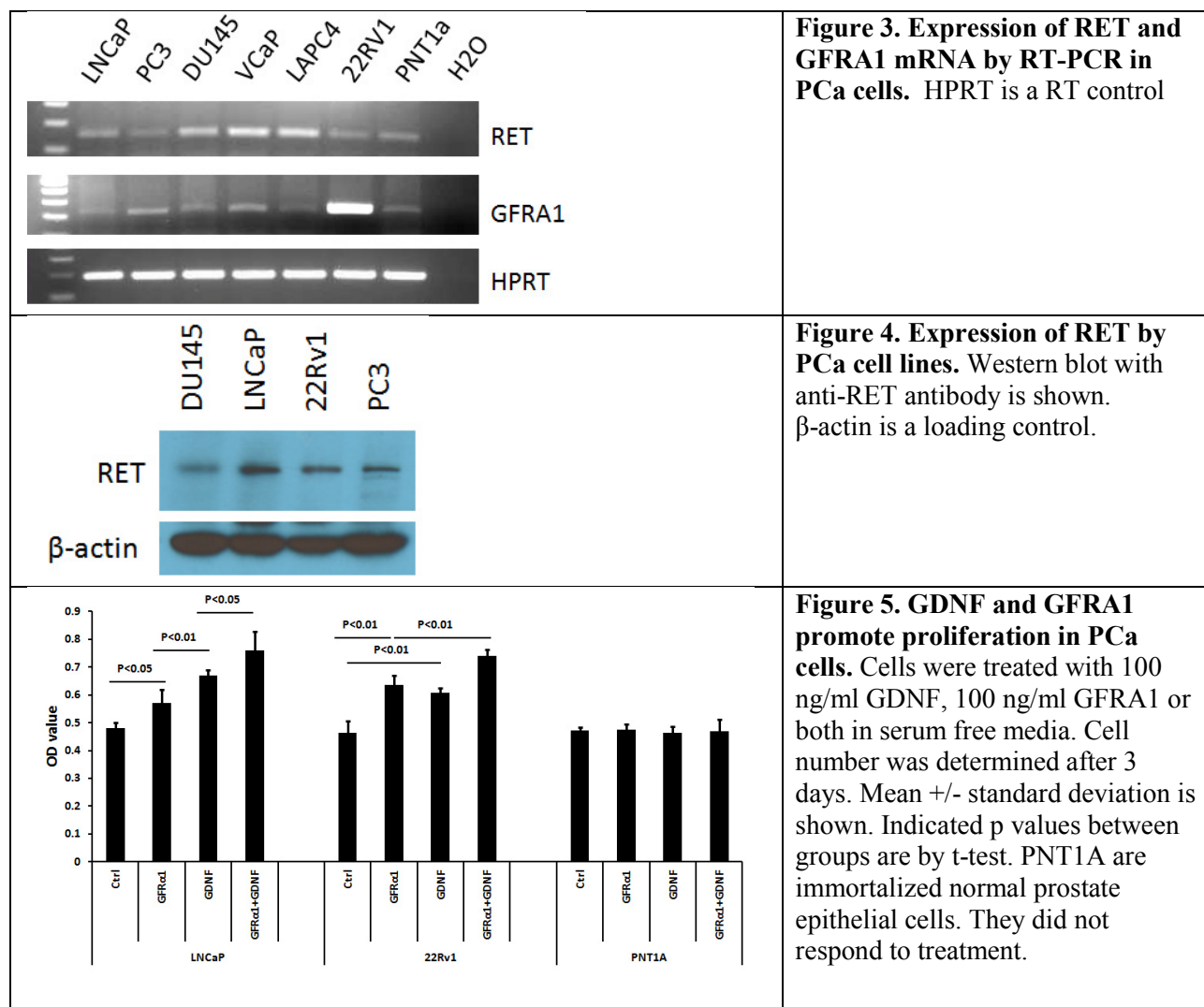
Major Task 2: In vitro studies of GDNF.

Subtask 1: **Sub-aim 1.1. Phenotypic affects of GDNF on prostate cancer cells in vitro (months 1-12)**

We have carried out a series of studies examining the ability of GDNF to promote neoplastic phenotypes in PCa cells in vitro. Stimulation of cells with GDNF resulted in increased invasion in VCaP cells (Fig 1) and increased pro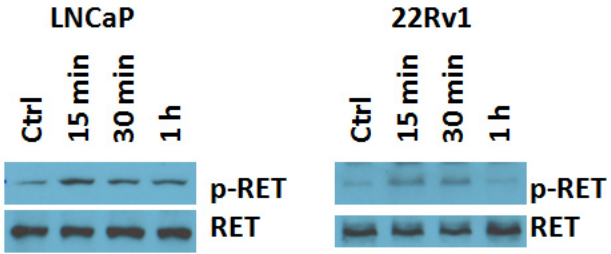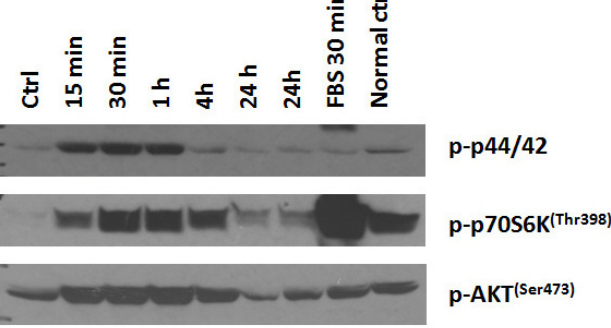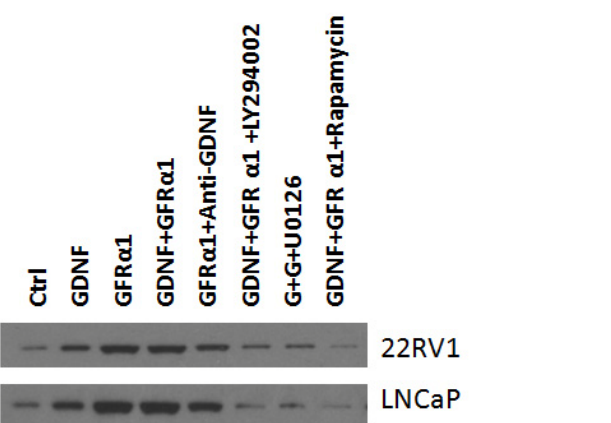liferation in LNCaP (Figure 2). PC3 also responded to GDNF with significant proliferation. However, DU145 (Fig 2) and VCAP (data not shown) did not respond to GDNF with proliferation. This result was initial puzzling since all PCa cell lines tested expressed RET mRNA by PCR (Fig 3). Western blot confirmed expression of RET protein at easily detectable levels in all cell lines (Fig 4). We therefore considered the possibility that while all the PCa cell lines tested express the RET co-receptor GFRA1 at levels detectable by PCR that perhaps this factor might be limiting for RET signaling. We therefore stimulated cells with GDNF, GFRA1 or both in combination. While GFRA1 has a short tail anchoring it to the cell membrane it also promotes RET signaling when added as a soluble protein. As seen in Figure 5, when added separately both GDNF and GFRA1 increase proliferation of PCa cell lines and together are more potent than either individually. Interestingly, immortalized benign prostate epithelial cells (PNT1A) do not respond to GDNF, GFRA1 or combination treatment. Overall our data indicates that exogenous GFRA1 is important for maximal stimulation of GDNF biological activities in PCa

Interestingly, new data has emerged from the pancreatic cancer literature relevant to our observations. He et al[2] have recently reported that soluble GFRA1 is released by nerves and plays a key role in enhancing nerve cancer interactions. We believe that a similar situation applies to PCa.

Figure1. Invasion is stimulated by GDNF in VCaP cells. Number of invasive cells is shown as mean +/-SEM. * $p<.05$, t-test versus control.

Figure 2. Stimulation of proliferation by GDNF. Cells were stimulated with the indicated concentration of GDNF in serum free medium for 3 days. Indicated p value is by t-test (0 Vs 100 ng/ml)

Figure 3. Expression of RET and GFRA1 mRNA by RT-PCR in PCa cells. HPRT is a RT control

Figure 4. Expression of RET by PCa cell lines. Western blot with anti-RET antibody is shown. β-actin is a loading control.

Figure 5. GDNF and GFRA1 promote proliferation in PCa cells. Cells were treated with 100 ng/ml GDNF, 100 ng/ml GFRA1 or both in serum free media. Cell number was determined after 3 days. Mean +/- standard deviation is shown. Indicated p values between groups are by t-test. PNT1A are immortalized normal prostate epithelial cells. They did not respond to treatment.

Subtask 2: Sub-aim 1.2. Pathway activation by GDNF: Western blotting (months 4-12)

We have undertaken a series of experiments to examine pathway activation in PCa cells after activation of RET signaling. As shown in Fig 6, RET signaling is activated with rapid kinetics after stimulation of PCa cell lines with GDNF and GFRA1. RE T phosphorylation increased within 15 minutes after stimulation. As seen in Figure 7, activation of RET signaling potently activates both ERK and AKT signaling. We noted that, as can be seen in Figures 7 and 8, p-70S6-kinase is potently activated by RET signaling in multiple cell lines. As can be seen from the inhibitor studies p70S6 kinase is downstream of both ERK and PI3-k signaling, which are both activated by RET signaling which accounts for the potent activation by RET signaling

	Figure 6 Activation of RET by GDNF and GFRA1. LNCaP or 22RV1 cells were placed in serum free medium overnight and stimulated with 100 ng ml GDNF and 100 ng/ml GFRA1 and cell lysates collected at the indicated times. Phosphorylated RET was detected by IP-Western. RET was detected by Western blot of input cell lysates
	Figure 7. Pathway activation by RET signaling in 22RV1 cells. Cell were placed in serum free medium overnight and cell lysates were collected at indicated times after activating RET signaling. Serum stimulation (FBS) was carried out for 30 min as a positive control. Normal control is cells growing in 10% FBS
	Figure 8. Activation of p70S6 kinase by GDNF and GFRA1. 22RV1 or LNCaP cells were placed overnight in serum free media and treated with 100 ng/ml of GDNF, GFRA1 or both and cell lysate collected. Controls included: neutralizing anti-GDNF antibody :5ug/ml, LY294002 (PI3-K inhibitor):20uM; U0126 (MEK inhibitor) :10uM, and Rapamycin (mTOR inhibitor): 1uM. Phosphorylated p70S6 kinase was detected by Western bot.

Subtask 3: Sub-aim 1.2. Pathway activation by GDNF: RPPA analysis (months 12-24)

We are currently planning this set of experiments with the Baylor College of Medicine Proteomics Core. They have a new and expanded printing of the Reverse Phase Proteomics Arrays (RPPA). The work in Subtask 1 and 2 above allows us to use optimal time points and cell lines for RPPA analysis to enhance our chances of discovering novel pathways downstream of RET signaling

Major Task 3: Role of GDNF in nerve-prostate cancer cell interactions.

Subtask 1: Sub-aim 2.1. DRG PNI studies with DU145 prostate cancer cells (months 4-15)

We have received assistance in setting up these experiments from the laboratory of Dr Gustavo Ayala at the University of Texas at Houston. Experiments are underway,

Subtask 2: Sub-aim 2.2. DRG PNI studies with other prostate cancer cell lines (months 12-36)

This will be carried beginning in Year 2

Subtask 3: Sub-aim 2.3. Role of RET in prostate cancer progression in vivo (months 4-36).

We have established multiple cell lines with RET knockdown for in vivo studies which are underway.

Major Task 3: In vivo expression of RET, GRFα1 and GDNF in prostate cancer..

Subtask 1: Immunohistochemistry for RET, GFRA1 and GDNF in human prostate cancer tissues including localized, metastatic and castrate resistant with quantitation (months 4-36)

We are currently optimizing immunohistochemistry conditions for these studies.

4. KEY RESEARCH ACCOMPLISHMENTS:
- We have shown that multiple prostate cancer cell lines can respond with proliferation and invasion to GDNF
- We have shown exogenous GFRA1 can potentiate GDNF biological responses in prostate cancer cells
- We have shown that GDNF can activate both ERK and AKT signaling in prostate cancer as well as downstream targets such as p70S6 kinase

5. CONCLUSION

Our studies show that GDNF can promote biological responses consistent with the transformed phenotype in PCa cells. These responses are potentiated by GFRA1. These studies suggest that GFRA1 may be partially limiting in our system. Moving forward we will need explore whether GFRA1 released by nerves may play an important in PCa-nerve interactions. RET signaling enhances both ERK and AKT signaling in PCa cells as well as downstream targets of these pathways such as p70S6 kinase. We are well positioned to proceed with DRG and in vivo mouse studies as well as immunohistochemistry of human tissues in the coming year.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS: None

7. INVENTIONS, PATENTS AND LICENSES: None

8. REPORTABLE OUTCOMES: None

9. OTHER ACHIEVEMENTS: None

10. REFERENCES

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2.	He S, Chen CH, Chernichenko N, Bakst RL, Barajas F, Deborde S, Allen PJ, Vakiani E, Yu Z, Wong RJ. GFRalpha1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(19):E2008-17. PMCID: 4024863.